REMARKS

Reconsideration is requested.

Claims 1, 2, 10, 43 and 44 are pending. Claim 10 has been amended to obviate the objection to the same and claims 1and 2 have been amended to clarify the recitation with regard to the fragment of the claims. No new matter has been added. The amendments are not believed to require further search and/or consideration. No new matter has been added. Entry of the amendments is requested.

The Section 112, first paragraph, rejection of claims 1, 2, 10, 43 and 44 is traversed. Reconsideration and withdrawal of the rejection are requested in view of the previously-submitted evidence as well as the following comments.

The applicants urge the Examiner to appreciate that the presently claimed invention relates to a method for inhibiting KDR/Flk-1 signal transduction in endothelial cells, and a method for inhibiting cell growth of endothelial cells, each of which involves contacting the cells with an anti-1175-tyrosine phosphorylated KDR/Flk-1 antibody (anti-PY1175 antibody.

The Examiner asserts that the previously-submitted evidence

> "require that the antibody be injected into the cell or transfected into the cell to achieve the observed effects. The techniques are not applicable for *in vivo*/therapeutic use in which the antibody must be able to contact antigen presented on the surface of the cell." See page 2 of the Office Action dated August 28, 2006.

The previously-submitted evidence (Declaration of Dr. Shitara submitted May 30, 2006) demonstrates that the cell growth of endothelial cells is inhibited by mixing the anti-PY1175 antibody with Chariot and contacting it with vascular cells. The Examiner

will appreciate that Chariot is also known as PEP-1 peptide having 21 amino acid residues, and is known to be a carrier peptide which transports a full-length protein *in vivo* and *in vitro*. See Abstract of attached Won Sik Eum et al, *Free Radical Biology & Medicine* Vol 37, No. 10, pp. 1656-1669 (2004)). The attached Eum et al further teaches that a fused polypeptide of antioxidant enzyme Cu,Zn-superoxide dismutase (SOD) and PEP-1 peptide is transduced *in vivo* in the skin (Fig 6) and in the neuronal cells of an ischemia animal model (Fig 7). The attached document is submitted as evidence in response to the Examiner's comments and as such is submitted to be timely made. See MPEP Section 609.05(c).

It will therefore be clear to one of ordinary skill in the art that cell growth of endothelial cells is inhibited *in vivo* by administering to an animal a mixture of anti-PY1175 antibody having an *in vitro* effect with the known PEP-1 peptide (Chariot).

The claims are submitted to be supported by an enabling disclosure and withdrawal of the Section 112, first paragraph, rejection of the claims is requested.

The objection to claim 10 is obviated by the above amendments. Entry of the present Amendment and withdrawal of the objection is requested.

The Section 112, first paragraph, rejection of claims 43 and 44 is obviated by the attached and the following. The applicants confirm by the attached Budapest Treaty deposit receipt that hybridoma KM3035 has been deposited as FERM BP-7729 under the provisions of the Budapest Treaty. The applicants confirm that all restrictions on the release of the deposited material will be removed upon the grant of the claims in a patent based on the present application. Withdrawal of the Section 112, first paragraph, rejection of claims 43 and 44 is requested.

The Section 112, first paragraph, rejection of claims 1, 2, 10, 43 and 44 is obviated by the above amendment. Entry of the present Amendment and withdrawal of the rejection are requested.

The claims are submitted to be in condition for allowance and a Notice to that effect is requested.

The Examiner is requested to contact the undersigned in the event anything further is required in this regard.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: /B. J. Sadoff/
B. J. Sadoff
Reg. No. 36,663

BJS:
901 North Glebe Road, 11th Floor
Arlington, VA 22203-1808
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

国際様式 INTERNATIONAL FORM

[特許手続上の微生物の寄託の国際的承認に関するブダペスト条約]

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

原寄託についての受託証

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

下記国際寄託当局によって規則7．1に従い発行される。

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

寄託者
氏名（名称） 協和醗酵工業株式会社
取締役社長 平田 正
あて名 〒 殿
東京都千代田区大手町一丁目6番1号

1．微生物の表示

(寄託者が付した識別のための表示)	(受託番号)
KM3.035	FERM BP- 7729

2．科学的性質及び分類学上の位置

1欄の微生物には、次の事項を記載した文書が添付されていた。

■ 科学的性質
■ 分類学上の位置

3．受領及び受託

本国際寄託当局は、平成 13 年 9 月 13 日（原寄託日）に受領した1欄の微生物を受託する。

4．移管請求の受領

本国際寄託当局は、 年 月 日（原寄託日）に1欄の微生物を受領した。
そして、 年 月 日に原寄託よりブダペスト条約に基づく寄託への移管請求を受領した。

5．国際寄託当局

独立行政法人産業技術総合研究所 特許生物寄託センター

名 称： International Patent Organism Depositary
National Institute of Advanced Industrial Science and Technology

センター長 小松 [illegible]
Dr. Yasuhiko Komatsu

あて名： 日本国茨城県つくば市東1丁目1番地1 中央第6（郵便番号 305-8566）
AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi
Ibaraki-ken 305-8566 Japan

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Original Contribution

IN VIVO PROTEIN TRANSDUCTION: BIOLOGICALLY ACTIVE INTACT PEP-1-SUPEROXIDE DISMUTASE FUSION PROTEIN EFFICIENTLY PROTECTS AGAINST ISCHEMIC INSULT

Won Sik Eum,* Dae Won Kim,* In Koo Hwang,† Ki-Yeon Yoo,† Tae-Cheon Kang,† Sang Ho Jang,* Heb Soon Choi,* Soo Hyun Choi,* Young Hoon Kim,* So Young Kim,* Hyeok Yil Kwon,‡ Jung Hoon Kang,§ Oh-Shin Kwon,¶ Sung-Woo Cho,‖ Kil Soo Lee,* Jinseu Park,* Moo Ho Won,† and Soo Young Choi*

*Department of Genetic Engineering, Research Institute for Bioscience and Biotechnology, †Department of Anatomy and ‡Department of Physiology, College of Medicine, Hallym University, Chunchon 200-702, Korea; §Department of Genetic Engineering, Chongju University, Chongju 360-764, Korea; ¶Department of Biochemistry, Kyungpook National University, Taegu 702-701, Korea; and ‖Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea

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Abstract—Reactive oxygen species (ROS) are implicated in reperfusion injury after transient focal cerebral ischemia. The antioxidant enzyme Cu,Zn-superoxide dismutase (SOD) is one of the major means by which cells counteract the deleterious effects of ROS after ischemia. Recently, we reported that denatured Tat-SOD fusion protein is transduced into cells and skin tissue. Moreover, PEP-1 peptide, which has 21 amino acid residues, is a known carrier peptide that delivers full-length native proteins in vitro and in vivo. In the present study, we investigated the protective effects of PEP-1-SOD fusion protein after ischemic insult. A human SOD gene was fused with PEP-1 peptide in a bacterial expression vector to produce a genetic in-frame PEP-1-SOD fusion protein. The expressed and purified fusion proteins were efficiently transduced both in vitro and in vivo with a native protein structure. Immunohistochemical analysis revealed that PEP-1-SOD injected intraperitoneally (i.p.) into mice can have access into brain neurons. When i.p.-injected into gerbils, PEP-1-SOD fusion proteins prevented neuronal cell death in the hippocampus caused by transient forebrain ischemia. These results suggest that the biologically active intact forms of PEP-1-SOD provide a more efficient strategy for therapeutic delivery in various human diseases related to this antioxidant enzyme or to ROS, including stroke.

Keywords—Protein transduction, PEP-1 peptide, Cu,Zn-SOD, Ischemia, Protein therapy, Free radicals

INTRODUCTION

Reactive-oxygen species (ROS) are inevitably formed as by-products of various normal cellular processes involving interactions with oxygen. These ROS damage macromolecules, which are constantly exposed to the harmful actions of ROS. Ultimately, these ROS contribute significantly to the pathological processes of various human diseases, which include ischemia, carcinogenesis, radiation injury, and inflammation/immune injury [1,2]. Cu,Zn-superoxide dismutase (SOD) is one of the key cellular enzymes that detoxifies intracellular free radicals, thus protecting them from oxidative damage [3].

Recently, several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous protein into living cells. These include carrier peptides derived from the HIV-1 Tat protein, *Drosophila* Antennapedia (Antp) protein, and herpes simplex virus VP22 protein [4–6]. Although the mechanism of protein transduction across the lipid bilayer is still unknown, it is clear that

Address correspondence to: Soo Young Choi, Department of Genetic Engineering, Hallym University, 1-1 Okchon-Dong, Chunchon, Kangwon-Do, 200-702, Korea; Fax: +82 33 241 1463; E-mail: sychoi@hallym.ac.kr.

transduction does not occur through a classical receptor or transporter or in an endosome-mediated fashion [7–9]. Up to the present, many researchers have demonstrated the successful delivery of full-length Tat fusion proteins by protein transduction technology. These include p27 [10], cdk2 [11,12], green fluorescent protein (GFP) [13], p53 [14], β-galactosidase (β-gal) [15], and so on. We previously reported that the genetic in-frame Tat–green fluorescent fusion protein (Tat-GFP), Tat–glutamate dehydrogenase (GDH), Tat–Cu,Zn-superoxide dismutase (Tat-SOD), and Tat–catalase (Tat-CAT) fusion proteins are efficiently transduced into mammalian cells and skin [16–26]. Transduced Tat-SOD was found to increase the viability of mammalian cells treated with paraquat, an intracellular superoxide anion generator, suggesting that it has a protective effect under conditions of oxidative stress [22,25]. Recently, we successfully transduced Tat-SOD directly into insulin-producing RINm-5F and islet cells across the lipid membrane under denaturing conditions [27]. In general, protein transduction using PTD-Tat fusion proteins required the denaturation of the fusion protein before delivery, which increases the accessibility of the Tat-PTD domain. After translocation of Tat-protein through the membrane in an unfolded state, members of the HSP90 protein family refold the target protein within the cell into an active conformation. Thus, the biological activity of the transduced protein was found to be dependent on the refolding efficacy of HSP90 protein [28].

To increase the biological activity of transduced protein in cells, a novel carrier is needed to transduce the target protein in its active native structure form. Recently, the Morris group [29] designed a PEP-1 peptide carrier (KETWWETWWTEWSQPKKKRKV), which consists of three domains: a hydrophobic tryptophan rich motif (KETWWETWWTEW), a spacer (SQP), and a hydrophilic lysine-rich domain (KKKRKV). When they mixed PEP-1 peptide and target protein (GFP, β-gal) and then overlaid on cultured cells, they found that the non-denatured target protein was transduced [29].

The antioxidant enzyme is one of the major mechanisms by which cells counteract the deleterious effects of ROS, and recent studies have revealed a protective effect of the antioxidant enzyme against apoptosis after cerebral ischemia and reperfusion. Also, they have shown that SOD plays a protective role against focal cerebral ischemia and is involved in regulation of cellular damage after ischemia/reperfusion [30–34].

In this study, we designed the PEP-1-SOD fusion protein by genetic in-frame for transduction directly in vitro and in vivo in its native active form and showed that the PEP-1-SOD fusion protein can be directly transduced into neuronal cells and across the blood–brain barrier and that it can efficiently protect against neuronal cell destruction. Therefore, we suggest that the PEP-1-SOD fusion protein would be useful as a potential therapeutic agent for transient forebrain ischemia.

MATERIALS AND METHODS

Expression and purification of PEP-1-SOD

PEP-1-SOD expression vector was constructed to express the PEP-1 peptides (KETWWETWW-TEWSQPKKKRKV) as a fusion with human Cu,Zn-SOD. First, Two oligonucleotides (top strand) 5′-TATGAAAGAAACCTGGTGGGAAACCTGGTGGA-CCGAATGGTCTCAGCCGAAAAAAAAACGTAAA-GTGC-3′ and (bottom strand) 5′-TCGABCACTTTAC-GTTTTTTTTTCGGCTGAGACCATTCGGTCCAC-CAGGTTTCCCACCAGGTTTCTTTCC-3′ were synthesized and annealed to generate a double-stranded oligonucleotide encoding the PEP-1 peptides. The double-stranded oligonucleotide was directly ligated into *Nde*I–*Xho*I-digested pET-15b vector. Second, on the basis of the cDNA sequence of human Cu,Zn-SOD, two primers were synthesized. The sense primer, 5′-CTCGAGGCGACGAAGGCCGTGTGCGTG-3′, contains an *Xho*I site, and the antisense primer, 5′-GGATCCTTATTGGGGCGATCCCAATTAC-3′, contains a *Bam*HI restriction site. PCR was performed [22] and the PCR product was excised with *Xho*I and *Bam*HI, eluted (Invitek, Berlin, German), ligated into TA-cloning vector (Promega, Madison, WI, USA) and pPEP-1 vector using T4 DNA ligase (Takaka, Otsu, Shiga, Japan), and cloned in *Escherichia coli* DH5α cells. PEP-1-SOD fusion proteins were generated when the human CuZn-SOD gene was fused with a 21-amino-acid PEP-1 peptide in a bacterial expression vector to produce a genetic in-frame PEP-1-SOD fusion protein.

To produce the PEP-1-SOD fusion protein, the plasmid was transformed into *E. coli* BL21 cells. The transformed bacterial cells were grown in 100 ml of LB medium at 37°C to a D_{600} value of 0.5–1.0 and induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 30°C for 12 h. Harvested cells were lysed by sonication at 4°C in a binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9), and the recombinant PEP-1-SOD formed was purified. Briefly, clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Valencia, CA, USA) under native conditions. After the column was washed with 10 volumes of the binding buffer and 6 volumes of a wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9), the fusion proteins were eluted using an eluting buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9). The fusion-protein-containing fractions were combined and the salts were removed using a PD-10 column

(Amersham, Braunschweig, Germany). The protein concentration was estimated by the Bradford procedure [35] using bovine serum albumin as a standard.

Transduction of PEP-1-SOD into astrocyte cells

For the transduction of PEP-1-SOD, astrocyte cells were grown to confluence on a six-well plate for 4–6 h. Then the culture medium was replaced with 1 ml of fresh solution. After astrocyte cells were treated with various concentrations of PEP-1-SOD for 30 min, the cells were treated with trypsin–EDTA (Gibco, Grand Island, NY, USA) and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts to perform a SOD enzyme assay and Western blot analysis.

The intracellular stability of transduced PEP-1-SOD fusion protein was estimated as follows. After astrocyte cells were treated with 2 μM native PEP-1-SOD for 1 h, the cells were washed and transferred to fresh culture medium to remove PEP-1-SOD that was not transduced. Then cells were further incubated for 72 h, followed by preparations of cell extracts for a SOD enzyme assay and Western blot analysis. The SOD enzyme assay and Western blot analysis were performed according to methods reported previously [22,27].

Immunofluorescecnce and fluorescence analysis

Immunofluorescence assay was performed using conjugated Cy-3 antibodies. Briefly, astrocyte cells were grown on glass coverslips and treated with PEP-1-SOD fusion protein. Following incubation for 30 min at 37°C, the cells were washed twice with trypsin–EDTA, PBS, and then fixed in 4% paraformaldehyde in 0.5 ml of PBS for 10 min at room temperature. The cells were washed with PBS and then incubated with polyhistidine antibody, following by incubation with Cy-3 antibody (1:1000) in PBS for 1 h.

For direct detection of fluorescein-labeled protein, purified PEP-1-SOD was labeled using EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit (Pierce, Rockford, IL, USA). The labeling with FITC was carried out according to the manufactuter's instructions. Cultured cells were grown on glass coverslips and treated with 2 μM PEP-1-SOD fusion protein. Following incubation for 30 min at 37°C, the cells were washed twice with PBS and trypsin–EDTA. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The distribution of fluorescence was analyzed on a fluorescence microscope (Carl Zeiss, EL-Einsatz, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The biological activity of the transduced PEP-1-SOD fusion proteins was assessed by measuring the viability of astrocyte cells treated with paraquat (methyl viologen; Sigma, St. Louis, MO, USA), which is well known as an intracellular superoxide dismutase anion generator. The cells seeded into six-well plates at 70% confluence. The cells were pretreated with 0.5–2 μM control SOD and native PEP-1-SOD for 1 h; then the 5 mM paraquat was added to the culture medium for 12 h. Cell viability was estimated with a colorimetric assay using MTT (Sigma).

Transduction of PEP-1-SOD into mice skin

To investigate the transductions of PEP-1-SOD fusion protein into mice skin, male ICR mice weighing about 30 g were used. The animals used in this experiment were treated according to the "Principles of Laboratory Animal Care" (NIH publication No. 86-23). Animals were anesthetized with 3% isoflurance in nitrogen and oxygen, 50 μg control SOD and PEP-1-SOD fusion proteins was topically applied onto the shaved area of the animal's skin for various time intervals. Thereafter, frozen and sectioned tissues were prepared and fixed with 4% paraformaldehyde for 10 min. For removal of nonspecific immunoreactivity, free-floating sections were first incubated with 0.3% Triton X-100 and 10% normal goat serum in PBS for 1 h at room temperature. They were then incubated with rabbit antihistidine IgG (1:500) for 24 h at room temperature. After washing three times for 10 min with PBS, the sections were incubated for 1 h with biotinylated goat antirabbit IgG (dilution 1:200; Vector Laboratories, Burlingame, CA, USA) and then visualized with 3,3′-diaminobenzidine (40 mg DAB/0.045% H_2O_2 in 100 ml PBS) mounted on gelatin-coated slides. The immunoreactions were observed under the Axioscope microscope (Carl Zeiss, Gottingen, Germany).

Induction of cerebral ischemia

Male Mongolian gerbils (*Meriones unguiculatus*) weighing 65–75 g were placed under general anesthesia with a mixture of 2.5% isoflurane (Baxtor, USA) in 33% oxygen and 67% nitrous oxide. To determine whether transduced PEP-1-SOD protects against ischemic damage, gerbils were i.p. injected PEP-1-SOD fusion protein in various concentrations (100–500 μg) at 30 min before or after the occlusion of both common carotid arteries. A midline ventral incision was made in the neck. Both common carotid arteries were isolated, freed of nerve fibers, and occluded with nontraumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in the eyeball using an ophthalmoscope. After 5 min occlusion, the aneurysm clips were removed. The restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope. Sham-operated animals (n = 7) were subjected to the same surgical procedures except that the common carotid arteries were not occluded. Rectal temperature was monitored and maintained at 37°C ± 0.5°C before,

during, and after the surgery until the animals had recovered fully from anesthesia. At 4 days after ischemia–reperfusion, the sham-operated group, vehicle-treated group, and PEP-1-SOD-treated group were euthanized for cresyl violet staining.

Effect of transduced PEP-1-SOD on neuronal cell viability after ischemic insult

All animals were anesthetized with pentobarbital sodium and perfused transcardially with PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) in the sham-operated group ($n = 7$), vehicle-treated group ($n = 7$), and PEP-1-SOD-pretreated and -posttreated groups at 4 days ($n = 7$, each) after the surgery. Brains were removed and postfixed in the same fixative for 4 h. The brain tissues were cryoprotected by infiltrating with 30% sucrose overnight. Thereafter, the tissues were frozen and sectioned with a cryostat at 30 μm and consecutive sections were collected in six-well plates containing PBS. These free-floating sections were then mounted on slide glass and air-dried. Thereafter, they were stained with cresyl violet acetate in the usual manner.

Quantitative analysis

For calculating the number of survival neurons, sections (10 sections per animal) representing the same level of the hippocampus were selected for measurement. Each studied field in each tissue was selected within the midpoint of the hippocampal CA1 region including all layers. Tissue images were obtained through an Axiophot light microscope (Carl Zeiss) connected via a charge-coupled device (CCD) camera to a PC monitor. Images of cresyl-violet-positive neurons were captured with an Applescanner. Quantification of the number of neuronal somata was performed using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, USA). Cell counts were obtained by averaging the counts from 70 sections taken from each animal. The number of cresyl-violet-positive neurons was compared to those of the sham-operated group. Finally, a Student t test was performed to investigate the protective effect of PEP-1-SOD [37].

RESULTS

Generation of a biologically active PEP-1-SOD fusion protein

To generate a cell-permeable expression vector, PEP-1-SOD vector, a human Cu,Zn-SOD cDNA was subcloned into the pET-15b plasmid that had been reconstructed to contain the PEP-1 peptide. The PEP-1-SOD expression vector formed contained consecutive cDNA sequences encoding human Cu, Zn-SOD, PEP-1 peptide (21 amino acids), and six histidine residues at the amino terminus. We also constructed the SOD expression vector (pSOD) to produce control SOD protein without PEP-1 transduction peptides (Fig. 1).

Expression and purification of PEP-1-SOD fusion protein

Following the induction of expression, PEP-1-SOD and control SOD fusion proteins were purified. Briefly, the fusion proteins were expressed in *E. coli* and clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column under native conditions. Fusion proteins containing fractions were combined and salts were removed using a PD-10 column. The crude cell extracts obtained from *E. coli* and purified PEP-1-SOD fusion proteins were electrophoresed in 15% SDS–PAGE. The expression and purification results are shown in Fig. 2A. The bands in lanes 2 and 3 show that the protein was highly expressed and was a major component of the total soluble proteins. In addition, the yields of the purified fusion proteins were approximately 70 mg/L on culture. The expressed and purified PEP-1-SOD enzyme activities

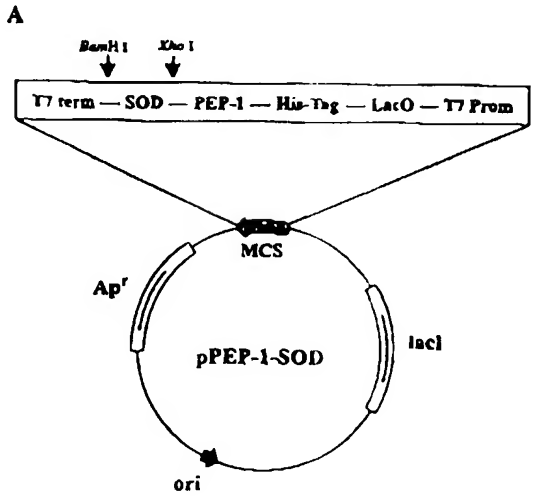

Fig. 1. Expression vector for PEP-1-SOD fusion protein. (A) Construction of the PEP-1-SOD expression vector system based on the vector pET-15b. Synthetic PEP-1 oligomer was cloned into the *NdeI*,*XhoI* sites, and human Cu,Zn-SOD cDNA was cloned into the *XhoI*,*BamHI* sites of pET-15b. (B) Diagram of expressed control SOD and PEP-1-SOD fusion proteins. The coding frame of human SOD is represented by an open box along with 6His and the PEP-1 peptide. The resulting vector was named pPEP-1-SOD. Expression was induced by adding IPTG.

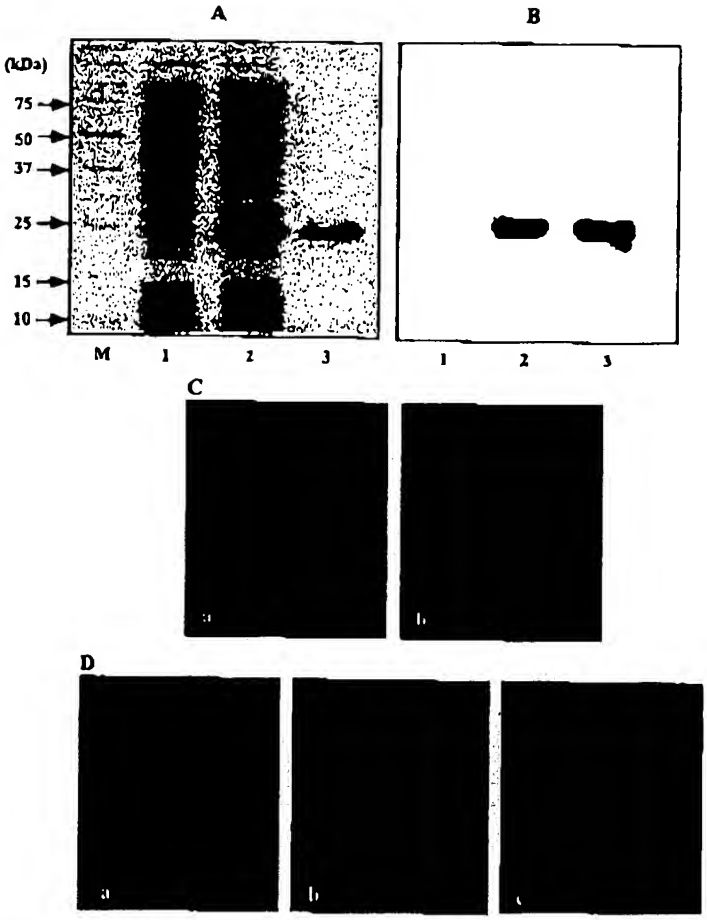

Fig. 2. Expression, purification, and cellular localization of PEP-1-SOD. Protein extracts of cells and purified fusion proteins were analyzed by 15% SDS-PAGE (A) and subjected to Western blot analysis with an anti-rabbit polyhistidine antibody (B). Lanes in A and B are as follows: lane 1, noninduced pPEP-1-SOD; lane 2, induced pPEP-1-SOD; lane 3, purified pPEP-1-SOD. Immunofluorescence and localization of transduced PEP-1-SOD proteins when the astrocyte cells were fixed or nonfixed with paraformaldehyde, respectively (C and D). Astrocyte cells were treated with 2 μM PEP-1-SOD fusion proteins for 30 min, and then the cells were incubated with anti-rabbit polyhistidine (1:400) and Cy-3 conjugated antibody (1:1000) for 1 h. Ca, control cells without PEP-1-SOD; Cb, cells treated with PEP-1-SOD. After FITC-labeled PEP-1-SOD protein (2 μM) was transduced into astrocyte cells, the cells were washed twice with trypsin–EDTA, PBS and immediately observed by fluorescence microscopy. Da, control cells without PEP-1-SOD; Db, nonfixed cells treated with PEP-1-SOD; Dc, fixed cells treated with PEP-1-SOD.

were similar to those of authentic human and recombinant Cu,Zn-SOD (data not shown) [22,38]. The expressed and purified proteins were further confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. PEP-1-SOD was detected at the corresponding bands in Fig. 2B. The control SOD expression and purification were as described previously (data not shown) [22].

Transduction of PEP-1-SOD fusion protein into astrocyte cells

To investigate the ability of PEP-1-SOD fusion protein to transduce into cells, it was conjugated to Cy-3 fluorescein (1:1000) and analyzed by immunofluorescence microscopy (Fig. 2C). As shown in Fig. 2C, native PEP-1-SOD fusion protein successfully transduced into the cells, whereas the control SOD did not transduce into the cells. These results indicate that PEP-1 peptide has a function and mechanism similar to those of HIV-1 Tat protein [4], the homeodomain of Antennapedia [5], and the herpes simplex virus VP22 protein [6], though the exact mechanism of protein transduction by PTD is unclear.

The intracellular delivery of PEP-1-SOD into astrocyte cells was further confirmed by direct fluorescence analysis. As shown in Fig. 2D, almost all cultured cells were found to be transduced with PEP-1-

SOD. To exclude the possibility that cell fixation with paraformaldehyde may affect PEP-1-SOD transduction by direct fluorescence, we used FITC-conjugated PEP-1-SOD fusion protein to transduce into nonfixed or fixed astrocyte cells. As shown in Fig. 2D, the intracellular distribution of the PEP-1-SOD fluorescence signal of nonfixed cells was similar to that of fixed cells. These results indicate that cell fixation with paraformaldehyde is not required for PEP-1-SOD fusion protein transduction.

To evaluate the transduction ability of PEP-1-SOD fusion protein, we analyzed the transduction of PEP-1-SOD proteins purified under native conditions by adding them to astrocyte culture medium at 2 μM for various times (5–30 min) and then analyzed the transduced protein levels by Western blotting. The intracellular concentration of transduced PEP-1-SOD in cells was detected within 5 min and was gradually increased at 30 min (Fig. 3A). The dose dependency of the transduction of PEP-1-SOD fusion proteins was further analyzed.

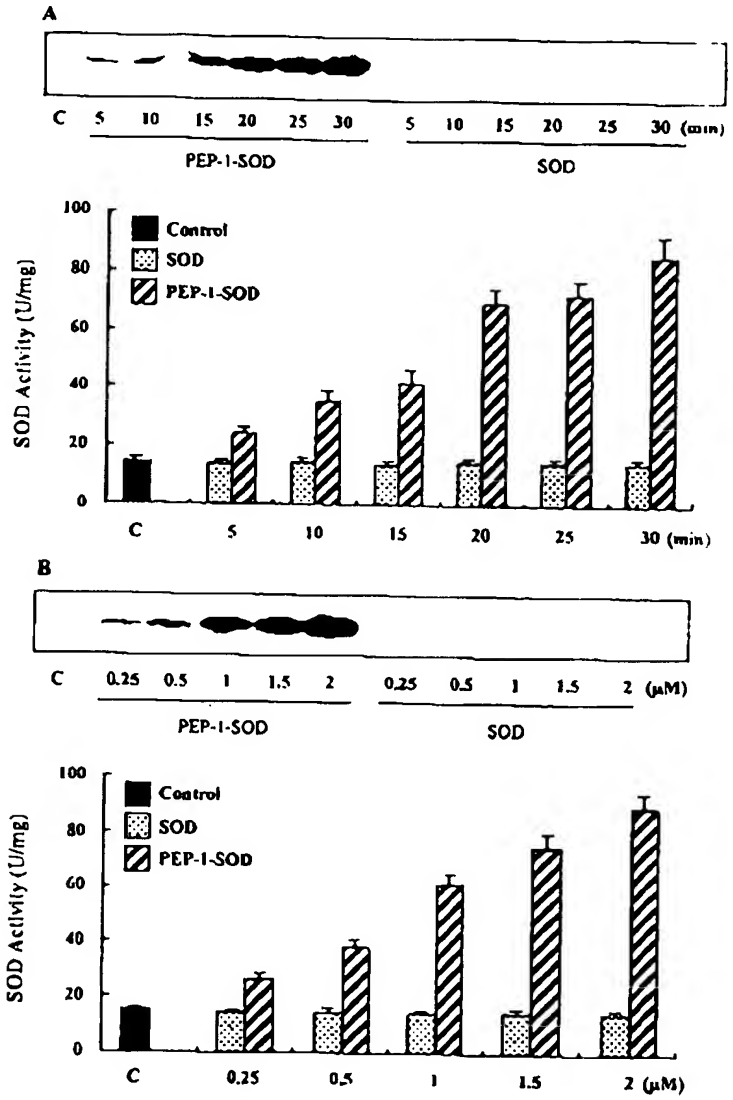

Fig. 3. Transduction of PEP-1-SOD fusion protein into cultured astrocyte cells. (A) 2 μM PEP-1-SOD and control SOD were added to the cultured media for 5–30 min, respectively. (B) 0.25–2 μM of PEP-1-SOD and control SOD were added to the cultured media for 30 min, respectively. Transduced PEP-1-SOD into cells was analyzed by Western blotting (top in A and B) and the specific enzyme activities (bottom in A and B), respectively. Each bar represents the mean ± SE obtained from five experiments

Various concentrations (0.25–2 μM) of PEP-1-SOD proteins were added to astrocytes in culture for 30 min, and the levels of transduced proteins were measured by Western blotting. As shown in Fig. 3B, the levels of transduced proteins in astrocyte cells were concentration-dependently increased on increasing the amount of fusion protein in the culture medium.

The properties of the transduced proteins in cells are key points for the therapeutic application of protein transduction. Therefore, we determined the dismutation activities of SOD in astrocytes treated with PEP-1-SOD (Figs. 3A and 3B, bottom). As shown at the bottom of Figs. 3A and 3B, the enzyme activity of SOD in transduced astrocyte cells increased in a time-and dose-dependent manner. The specific SOD enzyme activities were approximately two- to sevenfold increased by treating with various concentrations of PEP-1-SOD but not with the control SOD. However, the specific activity of SOD treated with 2 μM PEP-1-SOD was 88.76 U/mg.

The intracellular stability and enzymatic activity of PEP-1-SOD transduced into astrocyte cells are shown in Fig. 4. The PEP-1-SOD protein was added to the culture medium of astrocyte cells at a concentration of 2 μM for various time periods and the resulting levels of transduced protein were analyzed by Western blotting and enzyme activity. As shown in Fig. 4, the intracellular levels of transduced PEP-1-SOD into cells were initially detected after 1 h and then declined gradually over the period of observation. However, significant levels of transduced SOD and enzyme activities persisted in astrocyte cells for 48 h.

Effect of transduced PEP-1-SOD fusion protein on the viability of cells under oxidative stress

To determine whether the transduced fusion proteins have a functional role in cells, we tested the effects of transduced fusion proteins on cell viability under oxidative stress. The viability of cells treated with paraquat was significantly increased in a dose-dependent manner when pretreated with PEP-1-SOD. As shown in Fig. 5, when the cells were exposed to 5 mM paraquat without PEP-1-SOD, only 40–45% of cells were viable. However, the viability of the cells pretreated with PEP-1-SOD increased in a dose-dependent manner, reaching over 90% at the maximum concentration used. The increased viability of cells transduced with PEP-1-SOD suggests that this fusion protein has a critical protective effect against oxidative stress in cells.

Penetration of PEP-1-SOD fusion protein into mice skin

We then evaluated the ability of PEP-1-SOD fusion protein to transduce into mouse skin. PEP-1-SOD was sprayed onto mouse skin for 30 min and 1 h and the degree of penetration of these fusion proteins was analyzed immunohistochemically and by determining its enzymatic activity. As shown in Fig. 6A, transduction signals were significantly detected in the epidermis and dermis of skins treated with PEP-1-SOD fusion proteins. Unlike PEP-1-SOD, the control SOD could

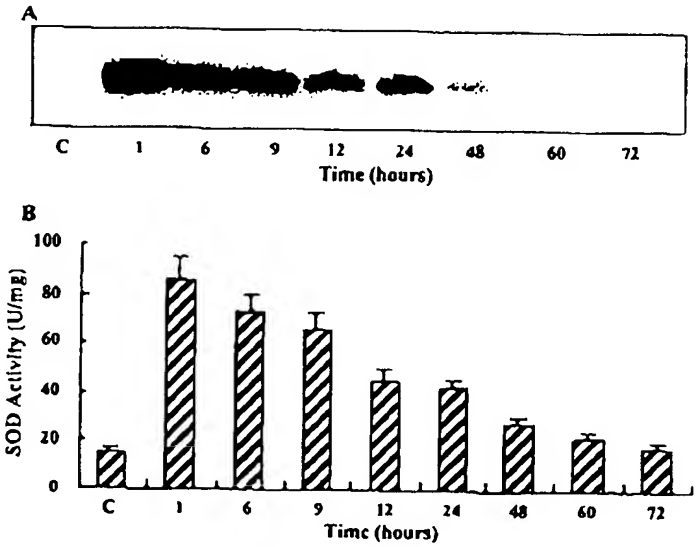

Fig. 4. Stability of PEP-1-SOD fusion protein transduced into cultured astrocyte cells. Cells pretreated with 2 μM PEP-1-SOD were incubated for various time periods (1–72 h). The PEP-1-SOD fusion protein transduced into cells was analyzed by Western blotting (A) and specific enzyme activities (B). Each bar represents the mean ± SE obtained from five experiments.

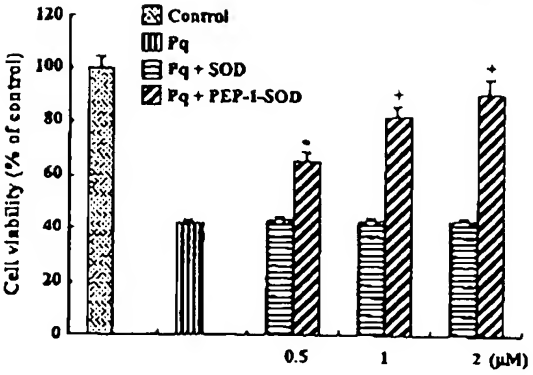

Fig. 5. Effect of transduced PEP-1-SOD on viability of astrocyte cells. The paraquat (Pq, 5 mM) was added to the astrocyte cells pretreated with 0.5–2 μM PEP-1-SOD and control SOD for 1 h. Cell viabilities were estimated with a colorimetric assay using MTT. Each bar represents the mean ± SE obtained from five experiments. Asterisks and crosses denote statistical significance at $p < 0.05$ and $p < 0.01$, respectively. The statistical comparisons were evaluated by Student's t test.

not transduce into skin. The levels of enzyme activities in skin were significantly increased when treated with PEP-1-SOD for 1 h. In untreated skin, the baseline level of total SOD activity was 4.9 ± 0.89 U/mg protein. However, skin that was treated with PEP-1-SOD contained levels of SOD activity that were approximately 6- to 10-fold (31.18 and 56.22 U/mg) time-dependently higher than the baseline (Fig. 6B). These results demonstrate that PEP-1-SOD fusion protein can not only be transduced into cultured cells but also penetrate the living animal skin.

In vivo transduced PEP-1-SOD fusion protein protects against ischemic damage

To determine whether transduced PEP-1-SOD perform their biological roles in vivo, we tested the effects of transduced PEP-1-SOD on neuronal cell viabilities after transient forebrain ischemia in a gerbil model. To determine whether transduced PEP-1-SOD protect aginast ischemic damage, we injected PEP-1-SOD fusion protein at various concentrations (100–500 μg) 30 min before or after ischemia. After 4 days after ischemic insult, the PEP-1-SOD-treated, vehicle-treated, and sham-operated control animals were killed for cresyl violet staining [36]. The protective effects of PEP-1-SOD fusion proteins after ischemic insult were evaluated by cresyl violet histochemistry (Fig. 7). In the vehicle-treated group, the percentage of the detected cresyl-violet-positive neurons was 11.2% compared to the sham-operated group (Figs. 7 and 8). PEP-1-SOD fusion proteins efficiently protected against neuronal cell damage caused by ischemic insult. In the PEP-1-SOD-treated group 4 days after ischemic insult, 85 to 92% neurons compared to the

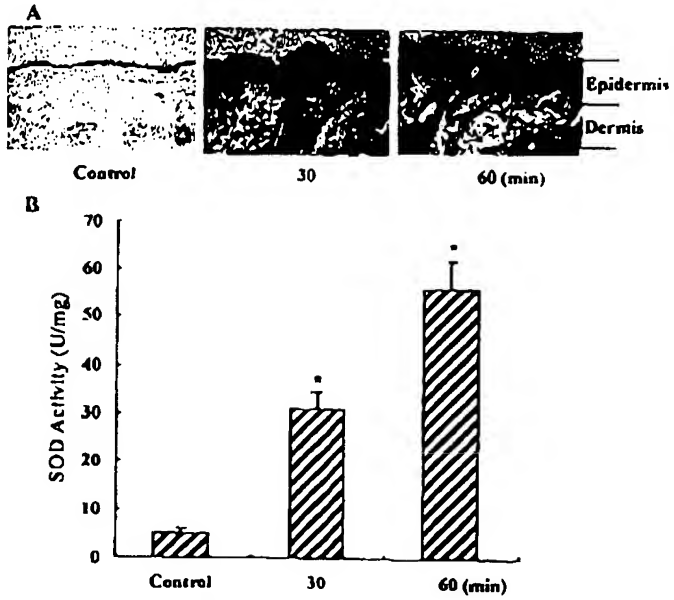

Fig. 6. Histochemical analysis of animal skin transduced with PEP-1-SOD fusion protein. PEP-1-SOD fusion proteins (50 μg)were topically applied onto the shaved area of mouse dorsal skin for 30–60 min. Frozen sections of skin tissues were immunostained with rabbit anti-histidine IgG (1:400) and then stained with biotinylated goat anti-rabbit IgG (1:200). The sections were visualized with 3,3'-diaminobenzidine and observed under an Axioscope microscope (A). Transduction efficiencies were analyzed by measuring specific enzyme activities in skin tissue (B). Each bar represents the mean ± SE for seven mice. Asterisks denote that the value is significantly different from that of the control group. The statistical analysis was evaluated by Student's t test.

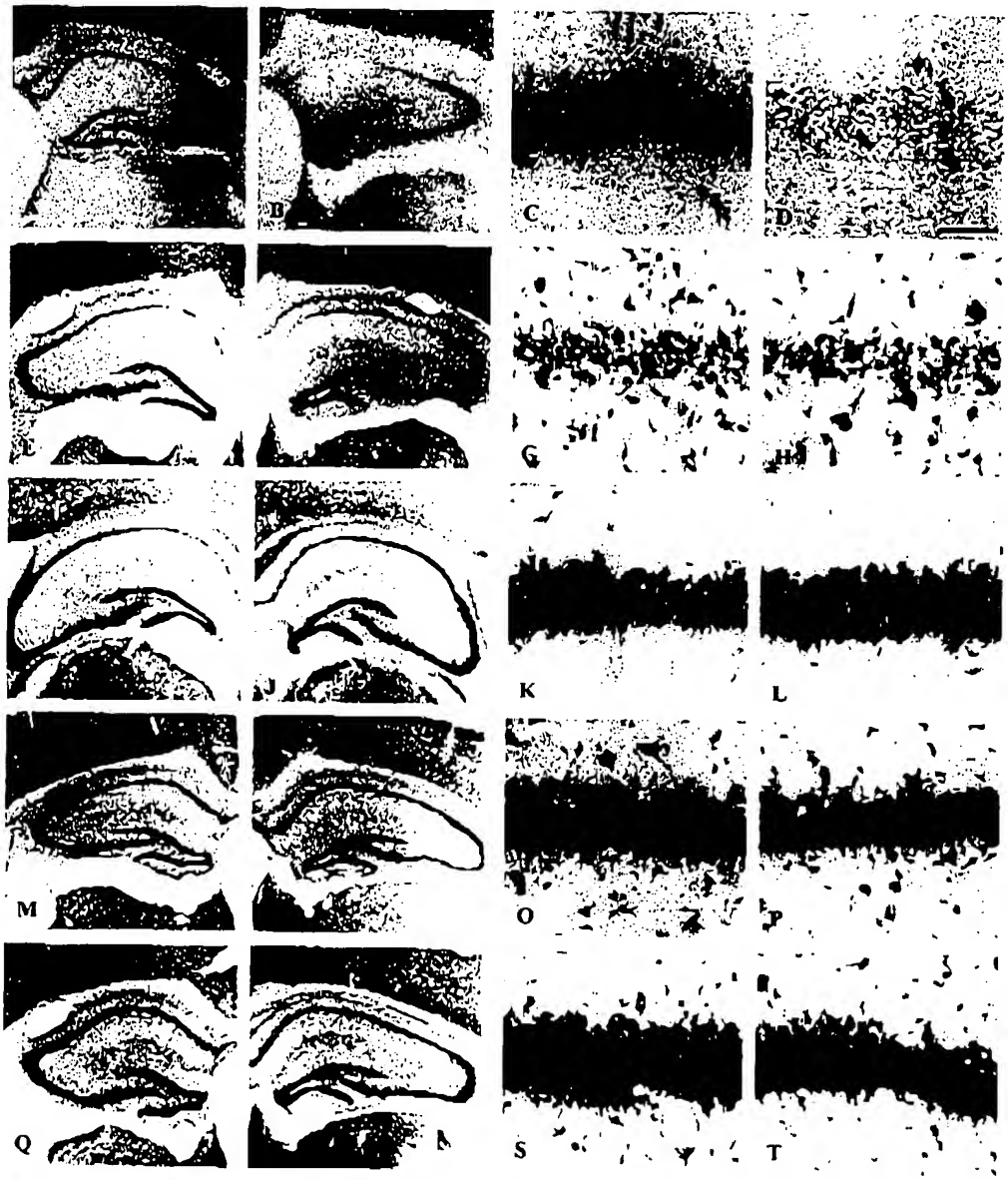

Fig. 7. Effects of transduced PEP-1-SOD on neuronal cell viability of ischemic insults. Representative photomicrographs of the cresyl violet-stained hippocampus of the gerbil brain 4 days after ischemic insults. Negative control (A and C; normal), positive control (B and D; vehicle-injected group), and PEP-1-SOD were pre-i.p. (E, G, I, K, M, O, Q, and S) or post-i.p. (F, H, J, L, N, P, R, and T) injected as a single dose at 100 μg (E–H), 200 μg (I–L) 300 μg (M–P), and 500 μg (Q–T) into the gerbil. Scale bars: A, B, E, F, I, J, M, N, Q, and R, 400 μm; C, D, G, H, K, L, O, P, S, and T, 50 μm.

sham-operated group were stained with cresyl violet (Figs 7 and 8). However, PEP-1-SOD-injected gerbils showed almost complete protection at low concentrations (200–300 μg) and PEP-1-SOD can play a major role in detoxifying ROS for at least 4 days after ischemia. These results indicate that PEP-1-SOD fusion proteins are efficiently transduced beyond the gerbil blood–brain barrier and effectively protect against neuronal cell death.

DISCUSSION

Although antioxidant enzymes have been considered potential therapeutic agents against reactive oxygen

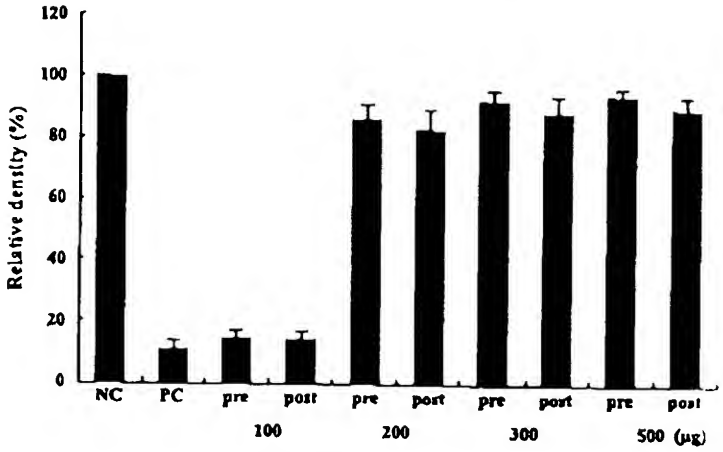

Fig. 8. Neuronal cell density in the hippocampal CA1 region of gerbils injected with PEP-1-SOD fusion protein. Negative control (NC; normal), positive control (PC; vehicle-injected group), and PEP-1-SOD fusion proteins were pre-or post-i.p. injected as a single dose at various concentrations (100–500 μg) into the gerbil after ischemic insult. Neuronal viabilities were higher in the groups treated with PEP-1-SOD than in the PC group. Each bar represents the mean ± SE obtained from seven gerbils.

species (ROS) mediated disease [39], the lack of transduction shown by antioxidant enzymes into cells has limited their use for protecting cells/tissues from oxidative damage. Therefore, in an effort to replenish the antioxidant enzyme activity in cells and tissues, we investigated the possibility of a protein transduction approach based on SOD enzyme into cell and tissues. Recently, the Morris group designed a 21-residue peptide carrier, PEP-1 (KETWWETWWTEWSQPKKKRKV), which consists of three domains: a hydrophobic tryptophan-rich motif (KETWWETWWTEW), a spacer (SQP), and a hydrophilic lysine-rich domain (KKKRKV). The hydrophobic domain is required for efficiently targeting the cell membrane and for hydrophobic interactions with proteins, whereas the hydrophilic domain is required to improve intracellular delivery. When mixed PEP-1 peptide and target protein (GFP, β-gal) were overlaid on cultured cells, it was found that the nondenatured target protein was transduced [29].

To develop an expression system capable of overexpressing the cell-permeable SOD protein, the human Cu,Zn-SOD gene was fused with a PEP-1 peptide in a bacterial expression vector to produce a genetic in-frame PEP-1-SOD fusion protein (Fig. 1). The expressed and purified PEP-1-SOD fusion proteins were confirmed by SDS–PAGE and Western blot using a monoclonal antibody to a human Cu,Zn-SOD (data not shown) and an anti-rabbit polyhistidine antibody (Figs. 2A and 2B). PEP-1-SOD fusion protein was highly expressed as a major component of the total soluble proteins in cells, and the purified protein yields were approximately 70 mg/L of culture under native conditions. In addition, the purified PEP-1-SOD fusion protein was found to be nearly homogeneous and greater than 95% pure, as determined by a SDS–PAGE analysis with Coomassie brilliant blue staining.

Our analysis of the transduction ability of the purified PEP-1-SOD fusion protein suggests that the native intact fusion protein was successfully delivered into the cells (Fig. 2C). In the case of HIV-1 Tat protein, native fusion protein was not delivered into the cells, as described previously [22]. However, this result indicates that, although the precise mechanism of protein transduction by PTD is unclear, the transduction mechanism of PEP-1 peptide may be similar to that of PTD in HIV-1 Tat protein [4,5], to that of PTD in the homeodomain of Antennapedia [6], and to that of PTD in the HSV-1 structural protein VP22 [40].

It is generally accepted that the Tat protein transduction domain of human immunodeficiency virus type 1 (HIV-1) efficiently transduces through the plasma membrane; although the mechanism of transduction is unclear, Tat PTD fusion protein transduction is one of the developments of protein therapeutics. A wide variety of functional proteins have been successfully used to study intracellular function and transduction into cells and tissues [41]. However, HIV-1 Tat PTD fusion protein transduction has been questioned [42,43], because it was demonstrated that HIV-1 Tat and $(Arg)_9$ PTD fusion protein transduction across the cell membrane does not occur in living cells and that it is an artifactual redistribution caused by cell fixation. The cell fixation technique disrupts the cell membrane and therefore cannot be reliably used to study membrane-translocating proteins. These peptides and fusion proteins are internalized into cells by endocytosis. Thus, it is argued that cell fixation should be avoided in

studies of protein transduction into living cells. However, in this study, we were unable to detect differences in the fluorescence distribution of transduced PEP-1-SOD in nonfixed and fixed cells (Fig. 2D). These results demonstrate that cell fixation with paraformaldehyde is not required for PEP-1-SOD transduction. Similar observations which indicate that artifacts of protein transduction are not induced by paraformaldehyde fixation have been reported, [29,44,45]. We also observed that the transduction of Tat-SOD fusion protein into insulin-producing cells (RINm-5F) by paraformaldehyde fixation did not have an effect [27].

Purified PEP-1-SOD fusion proteins efficiently transduced into cells in a time-and dose-dependent manner (Figs. 3A and 3B top). This time- and dose-dependent transduction indicates that PEP-1-SOD was rapidly transduced into cells. A recent study by Morris et al. [29] showed that PEP-1 peptide/GFP (30 kDa) or β-gal (119 kDa) mixtures transduce into a human fibroblast cell line (HS-68) and into Cos-7 cells by incubating with PEP-1 peptide carrier and proteins (GFP or β-gal) for 30 min at 37°C. These differences in the time courses of transduction may depend on whether the target protein is fused with the PEP-1 vector or mixed with the PEP-1 peptide. Because of fusion with the PEP-1 vector, the conformation, polarity, and molecular shape of a target protein might be altered, which improves the transduction of fusion proteins into cells.

The enzyme activity of transduced protein in cells is important for protein therapy application. Therefore, we determined the SOD activities in cells treated with PEP-1-SOD (Figs. 3A and 3B bottom). The level and enzyme activity of transduced PEP-1-SOD were found to be increased in a time-and dose-dependent manner. Transduced SOD enzyme specific activity increased up to six-fold (88.76 U/mg) that of control (14.7 U/mg). However, under the same conditions, Tat-SOD enzyme specific activity increased two- to threefold (data not shown). Transduced PEP-1-SOD enzyme activity is significantly higher than that of Tat-SOD. These differences in enzymatic activities of these fusion proteins imply that denatured Tat-SOD is needed for refolding after transduction, whereas PEP-1-SOD does not require refolding after transduction. Thus, PEP-1-SOD enzyme activities in the transduced cells were about three to four times higher than those of Tat-SOD. Morris et al. [29] showed that transduced β-gal had strong and uniform β-gal activity. In addition, the presence of PEP-1 did not alter the enzymatic activity of β-gal upon delivery into cells and was not affected by the presence of 10% fetal bovine serum or temperature (4°C), supporting that the protein transduction is independent of the endosomal pathway and has full enzyme activities [29].

As shown in Fig. 4, the intracellular stability of transduced PEP-1-SOD was significant with transduced protein and enzyme activity persisting in cells for 48 h. Under the same experimental conditions, the stability of Tat-SOD persisted for 24 h (data not shown). These results indicate that native PEP-1-SOD is more stable than denatured Tat-SOD. Therefore, we suggest that the transduced PEP-1-SOD offers a more powerful approach toward replenishing antioxidant enzyme activity in mammalian cells than HIV-1 Tat protein. To determine whether transduced PEP-1-SOD can play its biological role in the cells, we have tested the effect of transduced PEP-1-SOD on cell viability under oxidative stress. The viability of cells treated with paraquat was significantly increased when cells were pretreated with PEP-1-SOD fusion proteins in a dose-dependent manner (Fig. 5). After the cells were exposed to 5 mM paraquat without PEP-1-SOD, only 40–45% of the astrocyte cells were viable. However, the cell death was efficiently protected against by over 90% with transduced PEP-1-SOD. These results indicate that transduction of PEP-1-SOD was definitely effective against superoxide anion induced by paraquat in neuronal cells.

We then examined the ability of PEP-1-SOD fusion protein to transduce into mouse skin. As shown in Fig. 6, PEP-1-SOD fusion proteins were efficiently transduced into skin. The enzymatic activities of transduced PEP-1-SOD were more than 10-fold higher than those of control SOD. However, skin that was treated with Tat-SOD contained levels of SOD activity that were approximately three- to fourfold higher than those of the control SOD. Also, the control SOD without PTD could not penetrate into the skin [23]. These results demonstrate that PEP-1-SOD can be transduced more efficiently into mammalian cells and tissues and that it has potential use as a therapeutic agent against various disorders mediated by ROS.

To examine the ability of transduced PEP-1-SOD fusion protein to protect against ischemic damage, we designed a gerbil animal model. The formation of a large amount of toxic ROS in the hypoxic and ischemic brain has been proposed to be an important step in the sequence of events that links cerebral blood flow reduction to neuronal death [46,47]. ROS formation has been demonstrated during acute ischemic attack [46] and after blood and oxygen are eventually returned to the brain by reperfusion [48]. Several investigators have demonstrated that ROS-mediated toxicity affects virtually all cellular components in in vivo and in vitro ischemic models [49,50]. PEP-1-SOD was administered i.p. pre or post (30 min) ischemia. After 4 days the protective effects of the fusion proteins were confirmed by immunohistochemistry. As shown in Fig. 7, PEP-1-SOD fusion protein effectively protected against neuro-

nal cell death. In control gerbils, <12% of CA1 neurons survived the ischemic insult. The magnitude of the protective effect of SOD fusion proteins ranged from 85 to 92% survival for CA1 neurons. As shown in Fig. 8, PEP-1-SOD protected against neuronal cell death by up to 92%. In addition, at a low dose, PEP-1-SOD efficiently protected neurons by up to 85–90% after ischemia insult. These results indicate that native PEP-1-SOD efficiently protected against neuronal cell death.

Cu,Zn-SOD has been extensively used to reduce brain injury induced by ischemia and reperfusion. However, Cu,Zn-SOD was found to have a neuroprotective effect in transient focal cerebral ischemia in transgenic mice overexpressing SOD1 [51,52]. Recently, Chan et al. [53] demonstrated that in SOD1 transgenic rats cell damage was reduced in CA1 neurons by approximately 50% 3 days post ischemia. Damaged areas of CA1 neurons in SOD1 transgenic rats showed a significant protective effect against ischemia [53]. Thus, our results indicate that PEP-1-SOD fusion protein more efficiently protected against CA1 neuronal cell death than SOD1 gene therapy.

Morris et al. [29] reported that the PEP-1 peptide carrier presents several advantages for protein therapy, including the translocation of native protein, a high stability, a lack of toxicity, and a lack of sensitivity to serum. In particular, no toxicity to PEP-1 peptide was observed in several cell lines at up to 100–1000 μM, while cell viability was decreased by only about 10% for PEP-1 concentration of 1 mM.

Therefore, transduction of the antioxidant enzyme SOD fused with PEP-1 vector offers more attractive advantages for in vitro and in vivo protein therapy. Although the exact mechanisms of protein transduction are unclear, denaturation and the refolding of HIV-1 Tat PTD fusion protein are required for efficient transduction and physiologic activity, whereas the native PEP-1-SOD fusion protein does not need refolding in cells. In addition, the enzymatic activities of transduced PEP-1-SOD in cultured neuronal cells and mice skin are significantly increased and higher than those of Tat-SOD (data not shown).

In summary, we demonstrate for the first time that human Cu,Zn-SOD, a key antioxidant enzyme, fused with PEP-1 peptide (PEP-1-SOD) can be efficiently transduced in vivo and in vitro in its native conformation. Moreover, the transduced PEP-1-SOD was enzymatically and biologically active in cells and tissues. In addition, PEP-1-SOD significantly protected against neuronal cell death after treatment with paraquat and ischemic insult. Our success in the protein transduction of PEP-1-SOD may provide a new strategy for protecting against neuronal cell destruction resulting from ischemic damage and therefore may provide an opportunity for therapeutic intervention using antioxidant enzymes for the treatment of ROS-mediated diseases.

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REFERENCES

[1] Floyd, R. A. Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB J.* 4:2587–2597; 1990.
[2] Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press; 1999.
[3] Fridovich, I. Superoxide radical and superoxide dismutase. *Annu. Rev. Biochem.* 64:97–112; 1995.
[4] Fawell, S.; Seery, J.; Daikh, Y. Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. USA* 91: 664–668; 1991.
[5] Vives, E.; Brodin, P.; Lebleu, B. A. Truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272:16010–16017; 1997.
[6] Prochiantz, A. Messenger proteins: homeoproteins, TAT and others. *Curr. Opin. Cell Biol.* 12:400–406; 2000.
[7] Derossi, D.; Calvet, S.; Trembleau, A. Cell internalization of the third helix of the antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* 271:18188–18193; 1996.
[8] Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiantz, A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* 269: 10444–10450; 1994.
[9] Joliot, A.; Pernelle, C.; Deagostini-Bazin, H.; Prochiantz, A. Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl. Acad. Sci. USA* 88:1864–1868, 1991.
[10] Nagahara, H.; Vocero-Akbani, A. M., Snyder, E. L.; Ho, A.; Latham, D. G.; Lissy, N. A.; Becker-Hapak, M.; Ezhevsky, S. A.; Dowdy, S. T. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27^{Kip1} induces cell migration. *Nat. Med.* 4:1449–1452; 1998.
[11] Ezhevsky, S. A.; Ho, A.; Becker-Hapak, M., Davis, P. K.; Dowdy, S. F. Differential regulation of retinoblastoma tumor suppressor protein by G(1) cyclin-dependent kinase complexes in vivo. *Mol. Cell. Biol.* 21:4773–4783; 2001.
[12] Klekotka, P. K.; Santoro, S. A.; Ho, A., Dowdy, S. F.; Zutter, M. M. Mammaryepithelial cell-cycle progression via the $\alpha_2\beta_1$ integrin: unique and synergistic roles of the α_2 cytoplasmic domain. *Am. J. Pathol.* 159:983–992; 2001.
[13] Caron, N. J.; Torrente, Y.; Camirand, G.; Bujold, M.; Chapdelaine, P.; Leriche, K., Bresolin, N., Tremblay, J. P. Intracellular delivery of a Tat-eGFP fusion protein into muscle cells. *Mol. Ther.* 3:310–318; 2001.
[14] Wills, K. N.; Atencio, I. A.; Avanzini, J. B., Neuteboom, S.; Phelan, A.; Philopena, J.; Sugipto, S.; Vaillancourt, M. T.; Wen, S. F.; Ralston, R. O.; Johnson, D. E. Intratumoral spread and increased efficacy of a p53-VP22 fusion protein expressed by a recombinant adenovirus. *J. Virol.* 75:8733–8741; 2001
[15] Schwarze, S. R.; Ho, A.; Vocero-Akbani, A., Dowdy, S. R. *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* 285:1569–1572, 1999.
[16] Park, J.; Ryu, J.; Kim, K. A.; Lee, H. J., Bahn, J. H.; Han, K.; Choi, E. Y.; Lee, K. S.; Kwon, H. Y.; Choi, S. Y. Mutational analysis of a human immunodeficiency virus type 1 Tat protein transduction domain which is required for delivery of an exogenous protein into mammalian cells. *J. Gen. Virol.* 83:1173–1181; 2002a.

[17] Park, J.; Kim, K. A.; Ryu, J.; Choi, E. Y.; Lee, K. S.; Choi, S. Y. Generation and characterization of cell-permeable green fluorescent protein mediated by the basic domain of human immunodeficiency virus type 1 Tat. *J. Microbiol. Biotechnol.* 10:797–804; 2000.

[18] Ryu, J.; Han, K.; Park, J.; Choi, S. Y. Enhanced uptake of a heterologous protein with an HIV-1 Tat protein transduction domain (PTD) at both termini. *Mol. Cells* 16:385–391; 2003.

[19] Hahn, K.; Jeon, M. J.; Kim, S. H.; Ki, D. W.; Bahn, J. H.; Lee, K. S.; Park, J.; Choi, S. Y. Efficient intracellular delivery of an exogenous protein GFP with genetically fused basic oligopeptides. *Mol. Cells* 12:267–271; 2001.

[20] Hahn, K.; Jeon, M. J.; Kim, K. A.; Park, J.; Choi, S. Y. Efficient intracellular delivery of GFP by homeodomains of Drosophila Fushi-tarazu and engrailed proteins. *Mol. Cells* 10:728–732; 2000.

[21] Yoon, H. Y.; Lee, S. H.; Cho, S. W.; Lee, J. E.; Yoon, C. S.; Park, J.; Kim, T. U.; Choi, S Y. Tat-mediated delivery of human glutamate dehydrogenase into PC12 cells. *Neurochem. Int.* 41: 37–42; 2002.

[22] Kwon, H. Y.; Eum, W. S.; Jang, H. W.; Kang, J. H.; Ryu, J. Y.; Lee, B. R.; Jin, L. H.; Park, J.; Choi, S. Y. Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells. *FEBS Lett.* 485:163–167; 2000.

[23] Park, J.; Ryu, J.; Jin, L. H.; Bahn, J. H.; Kim, J. A.; Yoon, C. S.; Kim, D. W.; Han, K. H.; Eum, W. S.; Kwon, H. Y.; Kang, T. C.; Won, M. H.; Kang, J. H.; Cho, S. W.; Choi, S. Y. 9-Polylysine protein transduction domain: enhanced penetration efficiency of superoxide dismutase into mammalian cells and skin. *Mol. Cells* 13:202–208; 2002b.

[24] Kim, D. W.; Eum, W. S.; Jang, S. H.; Yoon, C. S.; Choi, H. S.; Choi, S. H.; Kim, Y. H.; Kim, S. Y.; Lee, E. S.; Back, N. I.; Kwon, H. Y.; Choi, J. H.; Choi, Y. C.; Kwon, O. S.; Cho, S. W.; Han, K.; Lee, K. S.; Park, J.; Choi, S. Y. Ginsenosides enhance the transduction of Tat-superoxide dismutase into mammalian cells and skin. *Mol. Cells* 16:402–406; 2003.

[25] Jin, L. H.; Eum, W. S.; Kwon, H. Y.; Bahn, J. H.; Jang, S. H.; Han, K. H.; Kang, T. C.; Won, M. H.; Kang, J. H.; Cho, S. W.; Park, J.; Choi, S. Y. Transduction of human catalase mediated by an HIV-1 Tat protein basic domain and arginine rich peptides into mammalian cells. *Free Radic. Biol. Med.* 31:1509–1519; 2001.

[26] Ryu, J.; Lee, H. J.; Kim, K. A.; Lee, J. Y.; Lee, K. S.; Park, J.; Choi, S. Y. Intracellular delivery of p53 fused to the basic domain of HIV-1 Tat. *Mol. Cells* 17:353–359; 2004.

[27] Eum, W. S.; Choung, I. S.; Li, M. Z.; Kang, J. H.; Kim, D. W.; Park, J.; Kwon, H. Y.; Choi, S. Y. HIV-1 Tat mediated protein transduction of Cu,Zn-superoxide dismutase into pancreatic β cells in vitro and in vivo. *Free Radic. Biol. Med.* 37:339–349; 2004.

[28] Schwarze, S. R.; Hruska, K. A.; Dowdy, S. F. Protein transduction: unrestricted delivery into all cells? Trends in Cell Biol. 10:290–295; 2000.

[29] Morris, M. C.; Depollier, J.; Mery, J.; Heitz, F.; Divita, G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat. Biotechnol.* 19:1173–1176; 2001.

[30] Murakami, K.; Kondo, T.; Kawase, M.; Li, Y.; Sato, S.; Chen, S. F.; Chan, P. H. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J. Neurosci.* 18:205–213; 1998.

[31] Fujimura, M.; Morita-Fujimura, Y.; Narasimhan, P.; Copin, J.-C.; Kawase, M.; Chan, P. H. Copper-zinc superoxide dismutase prevents the early decrease of apurinic/apyrimidino endonuclease and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. *Stroke* 30:2408–2415; 1999.

[32] Fujimura, M.; Morita-Fujimura, Y.; Noshita, N.; Sugawara, T.; Kawase, M.; Chan, P. H. The cytosolic antioxidant copper/zinc-superoxide dismutase prevents the early release of mitochondrial cytochrome c in ischemic brain after transient focal cerebral ischemia in mice. *J. Neurosci.* 20:2817–2824; 2000.

[33] Noshita, N.; Sugawara, T.; Fujimura, M.; Morita-Fujimura, Y.; Chan, P. H. Manganese superoxide dismutase affects cytochrome c release and caspase-9 activation after transient focal cerebral ischemia in mice. *J Cereb. Blood Flow Metab.* 21:557–567; 2001.

[34] Sugawara, T.; Noshita, N.; Lewen, A.; Gasche, Y.; Ferrand-Drake, M.; Fujimura, M.; Morita-Fujimura, Y., Chan, P. H. Overexpression of copper/zinc superoxide dismutase in transient rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. *J. Neurosci.* 22:209–217; 2002.

[35] Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254; 1976.

[36] Won, M. H.; Kang, T. C.; Park, S. K., Jeon, G. S.; Kim, Y. W.; Seo, J. H.; Choi, E. M.; Chung, M. H., Cho, S. S. The alterations of N-Methyl-D-aspartate receptor expressions and oxidative DAN damage in the CA1 area at the early time after ischemic-reperfusion insult. *Neurosci. Lett.* 301:139–142; 2001.

[37] Kang, T. C.; Park, S. K.; Hwang, I. K.; An, S. J.; Choi, S. Y.; Cho, S. W.; Won, M. H. Spatial and temporal alterations in the GABA shunt in the gerbil hippocampus following transient ischemia. *Brain Res.* 944:10–18; 2002.

[38] Kang, J. H.; Choi, B. J.; Kim, S. M Expression and characterization of recombinant human Cu,Zn-superoxide dismutase in *Escherichia coli*. *J. Biochem. Mol. Biol.* 30:60–67; 1997.

[39] Greenwald, R. A. Superoxide dismutase and catalase as therapeutic agents for human diseases. A critical review. *Free Radic. Biol. Med.* 8:201–209; 1990.

[40] Chen, Y. N.; Sharma, S. K.; Ramsey, T. M.; Jiang, L.; Martin, M. S.; Baker, K.; Adams, P. D.; Bair, K. W.; Kaelin, W. G., Jr. Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. *Proc. Natl. Acad. Sci. USA* 96:4325–4329; 1999.

[41] Wadia, J. S.; Dowdy, S. F. Protein transduction technology. *Curr. Opin. Biotechnol.* 13:52–56; 2002.

[42] Lundberg, M.; Wikstrom, S.; Johansson, M. Cell surface adherence and endocytosis of protein transduction domains *Mol. Ther.* 8:143–150; 2003.

[43] Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. Cell-permeable peptides: A reevaluation of the mechanism of cellular uptake. *J Biol. Chem.* 278:585–590; 2003

[44] Cashman, S. M.; Morris, D. J.; Kumar-Singh, R. Evidence of protein transduction but not intracellular by protein fused to HIV Tat in retinal cell culture and in vivo. *Mol. Ther.* 8:130–142; 2003.

[45] Brewis, N.; Phelan, A.; Webb, J.; Drew, J., Elliott, G.; O'hare, P. Evaluation of VP22 spread in tissue culture. *J. Virol.* 74: 1051–1056; 2000.

[46] Demopoulos, H. B.; Flamm, E. S.; Pietronigro, D. D.; Seligman, M N. The free radical pathology and the microcirculation in the major central nervous system disorders. *Acta Physiol. Scand.* 492: S91–S119; 1980.

[47] Fujita, H.; Sato, K.; Wen, T. C., Peng, Y.; Sakanaka, M. Differential expression of glycine transporter 1 and three glutamate transporter mRNA in the hippocampus of gerbils with transient forebrain ischemia. *J. Cereb. Blood Flow Metab.* 19:604–615; 1999.

[48] Hall, E. D.; Braughler, J. M. Central nervous system trauma and stroke. II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation. *Free Radic. Biol. Med.* 6:303–313; 1989.

[49] Liu, P. K.; Hsu, C. Y.; Dizdaroglu, M.; Floyd, R. A.; Kow, Y. W.; Karakaya, A.; Rabow, L. E.; Cui, J. K. Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. *J. Neurosci.* 16:6795–6806; 1996.

[50] McCord, J. M. Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* 312:159–163; 1985.

[51] Kinouchi, H.; Epstein, C. J.; Mizui, T.; Carlson, E. J.; Chen, S. F.; Chan, P. H. Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing Cu,Zn-superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 88:11158–11162; 1991.

[52] Yang, G.; Chan, P. H.; Chen, J.; Carlson, E.; Chen, S. F.; Weinstein, P.; Epstein, C. J.; Kamii, H. Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. *Stroke* 25:165–170; 1994.

[53] Chan, P. H.; Kawase, M.; Murakami, K.; Chen, S. P.; Li, Y.; Calagui, B.; Reola, L.; Carlson, E.; Epstein, C. J. Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion. *J Neurosci.* 18:8292–8299; 1998.